

**A CONNECTION BETWEEN PRIMARY LYMPHEDEMA
AND CYSTIC HYGROMAS?**

by

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ABSTRACT

The lymphatic system is a network of organs and vessels that serve different purposes including the filtering of blood and the initiation of an immune response to an infection. Lymphedema is the swelling of the arm(s) and/or leg(s) from removal, damage, or blockage of the lymphatic system. Lymphedema can occur as a secondary condition caused by another disease or condition, but lymphedema can also be inherited. Cystic hygroma is a collection of lymphatic fluid at the back of the neck that may be observed during a prenatal ultrasound exam and then disappear on its own, although sometimes it is still present at birth. Some evidence suggests that one cause of cystic hygroma is inherited lymphedema. Inherited lymphedema and cystic hygromas are findings that may require treatment and follow-up. If an association exists between cystic hygromas and inherited lymphedema, then establishing this connection can help predict prognosis and provide appropriate care at both the individual level and the population level for public health significance.

An introduction letter and cystic hygroma questionnaire were sent out to the participants of the University of Pittsburgh's Lymphedema Family Study who have voluntarily provided their own biological samples previously (1,628 people). The questionnaire was completely voluntary and was either mailed or faxed back when completed. It inquired about lymphedema, cystic

hygromas, and prognosis. It also inquired about other biological family members including babies that did not survive to birth. Follow-up phone calls were made to some families for clarification.

Results show that 316 (19.4%) research participants have completed and returned the questionnaire, 2.44% of those that returned the questionnaire and have lymphedema also had cystic hygromas, and 0.57% of those without lymphedema that returned the questionnaire had cystic hygromas. This difference in cystic hygroma prevalence is not statistically significant ($p = 0.3094$). However, there is some enough evidence to support the claim that there is a higher prevalence of cystic hygromas in those with lymphedema compared to the general population (18.3-fold difference). It is possible that the number of individuals diagnosed with cystic hygromas is underreported because prenatal ultrasounds were not available for many participants.

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PREFACE

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1.0 INTRODUCTION

The lymphatic system is a network of organs and vessels that serve different purposes including aiding the immune system. Lymphedema is the swelling that occurs typically in the arms and legs due to blockage, damage, or malformation of the lymphatic vessels. Primary lymphedema is an inherited condition, but may have an unknown cause. Secondary lymphedema is caused by an external process such as surgery, infection, radiation, or cancer. Cystic hygromas are fluid-filled growths that typically occur in the neck or head area due to partial malformation of the lymphatic system. Cystic hygromas are found prenatally via ultrasound, at birth, or within the first couple years of life. Fifty to sixty percent of cystic hygroma cases also have a chromosomal condition that can be detected by karyotype.

The Lymphedema Family Study is a University of Pittsburgh study designed to serve and do research on families with lymphedema and has been active since 1995. There were 2,229 biological sample kits and surveys sent out for the Lymphedema Family Study. The survey has identified 7 individuals that have both lymphedema and cystic hygromas (plus one individual with lymphedema that may have had a cystic hygroma) through the free-response section of the survey. The purpose of this current study is to explicitly investigate a possible association between primary lymphedema and cystic hygromas with the questionnaire shown in Appendix B and to help establish prognosis for those with both primary lymphedema and cystic hygromas. If

an association exists between primary lymphedema and cystic hygromas, then this knowledge can help predict prognosis and provide appropriate care.

1.1 AIMS

Aim 1: To ascertain the proportion of people with cystic hygromas of those with and without lymphedema.

Aim 2: To determine if a statistically significant difference exists in cystic hygroma prevalence between those with and without lymphedema.

Aim 3: To ascertain the prognosis of people with cystic hygromas and lymphedema.

1.2 BACKGROUND AND SIGNIFICANCE

1.2.1 Lymphatic System

The lymphatic system is a network of organs and vessels that serves different functions. One function of this system is the collection of excess interstitial fluid that was not absorbed by the surrounding tissues or the vascular network. Lymph is the fluid that travels through the lymphatic system. Muscle contractions provide the force necessary to move the lymph fluid through the lymphatic system to ultimately return this fluid to the circulatory system. Before this can happen though, the lymph must travel through the lymph nodes which filter the lymph for

damaged cells, debris, waste, foreign agents, and cancer cells. These lymph nodes contain different white blood cells including lymphocytes for this purpose. Therefore, the lymphatic system is not only involved in transport, it is also involved in aiding the immune system [1, 2].

1.2.2 Lymphedema

Lymphedema is the swelling usually in the arm(s) and/or leg(s) from partial malformation, removal, blockage, or damage of the lymphatic system [3]. It is estimated that 0.133% - 0.144% of the population has lymphedema, but this could be an underestimate due to lack of diagnosis or misdiagnosis [4-6]. Complications can occur due to lymphedema such as heaviness or tightness feelings in the extremities. The range of motion can be restricted. Pain, aching, discomfort, and pressure sensations could develop. A person with lymphedema is at an increased risk of infections including bacterial skin infections (cellulitis) and infection of the lymph vessels themselves (lymphangitis). Affected skin can become hardened. Untreated lymphedema can lead to a rare form of cancer called lymphangiosarcoma [3]. Some people with lymphedema can also have a negative body image.

Although there is no cure for lymphedema, different treatments are available. Light exercise can help drain excess lymph. Wrapping the affected arm or leg can help the lymph flow from the limb to the trunk of the body. Manual lymph drainage is a special type of massage performed by a trained expert to help flow the lymph to the trunk of the body. Not everyone with lymphedema should have this done. Exclusion criteria include: skin infection, cancer, congestive heart failure, blood clots, and regions of the body that have received radiation therapy. Compression of the affected arm or leg with a compression garment or with an intermittently-inflating sleeve is another treatment option. Ultimately, the goal is to help move

the lymph out of the affected extremities and to the trunk of the body. Surgery to alleviate the swelling is another option; however, surgery can also exacerbate swelling if damage to the lymphatic vessels or lymph nodes occurs [3].

There are two types of lymphedema, primary and secondary. Primary lymphedema is a genetic or inherited condition that causes developmental problems with lymph vessel formation or function. Secondary lymphedema is caused by external factors such as infection, cancer, radiation, and surgery. A classic example of secondary lymphedema is surgical removal or injury to lymph nodes and lymphatic vessels to check for the spread of breast cancer. Radiation treatment for cancer can also damage lymphatic vessels leading to lymphedema. Cancer itself or certain infections such as parasites can block lymphatic vessel(s) which then causes swelling. Secondary lymphedema caused by infections are more common in tropical areas of the world, especially in developing countries [3].

1.2.3 Genetic Causes of Primary Lymphedema

The prevalence of primary lymphedema for those younger than 20 years old may be 1.15 out of 100,000 [4, 7]. Congenital lymphedema is present within the first two years of life. Lymphedema praecox typically appears around the age of puberty, but can be present for someone in his/her 20s. Lymphedema tarda usually develops after age 35 [4].

When a hereditary pattern of primary lymphedema is present in a pedigree, it most likely follows an autosomal dominant pattern of inheritance; however, autosomal recessive inheritance has also been reported [4]. One important example of autosomal dominant primary lymphedema is lymphedema-distichiasis (LD) syndrome [4, 8]. This syndrome typically manifests during

puberty and involves distichiasis which means that extra eyelashes (sometimes an entire row) form posterior to the lid margins. These eyelashes tend to point downward and can cause severe damage to the cornea. Distichiasis is 94.2% penetrant in most LD families. Ptosis (31%), congenital heart disease (7%), cleft palate (4%), spinal extradural cysts, and early onset varicose veins (49%) have also been reported in association with lymphedema-distichiasis syndrome [4, 8]. The only known genetic causes of LD syndrome are mutations in the *FOXC2* gene. *FOXC2* is a forkhead transcription factor gene [8].

Mutations in the FMS-Related Tyrosine Kinase 4 (*FLT4*) gene (also known as *VEGFR3*) will yield an autosomal dominant congenital primary lymphedema condition called Milroy disease [5, 8]. In a study by Brice et al., 90% of those with a mutation in this gene developed lower extremity lymphedema. Swelling was present at birth except for two cases. Cellulitis (20%), large leg veins (23%), upslanting toenails (10%), and development of papillomas (10%) are associated with Milroy disease. For men, hydrocele (37%) was the most prevalent finding besides lymphedema [9].

Single gene mutations are not the only genetic cause of primary lymphedema, which can also occur in individuals with Turner syndrome. It is estimated that 17% - 30% of those with Turner syndrome have lymphedema [10-12]. This is a condition that affects development in females and it is estimated that 1/2,500 female births have Turner syndrome, which is even more common among miscarriages and stillbirths. Women with Turner syndrome tend to be of short stature and infertile due to lack of ovarian function. They also exhibit a webbed neck, skeletal anomalies, heart problems, and kidney problems. Women with Turner syndrome do not have two complete X chromosomes. Having only one copy of the *SHOX* gene (located on the X chromosome) probably causes the skeletal issues associated with Turner syndrome [13].

Other symptoms of Turner syndrome include frequent middle ear infections and potential deafness during childhood. Some girls may have problems with memory skills, fine motor skills, and math. Females with this condition can have a broad chest with widely-spaced nipples. Arms may turn out slightly from the elbow. High blood pressure, scoliosis, and minor eye problems are also possible. Hypothyroidism, slight risk of diabetes, and osteoporosis are also concerns [13].

It has also been shown that Turner syndrome can lead to cystic hygromas [14].

1.2.4 Cystic Hygromas

Cystic hygromas are fluid-filled growths that typically occur in the neck or head area due to partial malformation of the lymphatic system. They can be detected via ultrasound during pregnancy, at birth, or within the first two years of life [15]. Cystic hygromas in the neck region occur in 1/6,000 live births and 1/750 miscarriages [14]. Some cystic hygromas resolve during pregnancy; however, 85% of cystic hygromas grow larger than the fetus's head. Cystic hygromas can cause: extra folds of skin at the neck (webbed neck), some tissue swelling, non-malignant tumors on the skin known as lymphangiomas, and hydrops where there is too much fluid within the baby's body. Hydrops is very serious and can lead to miscarriage or death [15].

Treatment options for cystic hygromas include monitoring, surgery, and sclerotherapy. Surgical removal of the fluid-filled cysts is one treatment option. It should be noted that a cystic hygroma may come back after surgery in 10% - 15% of cases. Sclerotherapy is another treatment option that does not involve surgery. Ultrasound is used to guide the needle into the

cyst. Medicine is injected which causes the lymphatic vessels and the cyst itself to decrease in size [15].

Cystic hygromas can cause hydrops as well as cosmetic concerns. It is important to note that additional complications, such as infections, can arise. Sometimes the cystic hygroma will form an abscess which needs to be drained. Antibiotics, antipyretics, and analgesics can be used to help treat the infected cystic hygroma. Cystic hygromas can spontaneously bleed. The cysts can become very hard when this occurs, and surgery may be required in these situations. Respiratory difficulties and trouble swallowing can also occur if the cystic hygroma is in the neck and/or oral cavity [16].

Environmental causes of cystic hygroma include maternal viral infections and fetal alcohol syndrome [15]. There are also different genetic causes for cystic hygromas including Turner syndrome [14]. However, the cause of cystic hygroma is often unknown.

1.2.5 Genetic Causes of Cystic Hygromas

Between 50-60% of cystic hygroma cases involve an abnormal karyotype or genetic syndrome [14, 15, 17, 18]. Turner syndrome and Trisomy 18 each contributed approximately 15.4% to the cause of cystic hygromas according to Tanriverdi et al. [14]. However, Hoswarth et al. showed in his study that Turner Syndrome is the most common chromosomal cause of cystic hygromas with 33% of pregnancies with cystic hygromas having Turner Syndrome [18]. Other genetic conditions associated with cystic hygromas include: trisomy 21 (Down syndrome), trisomy 13, Noonan syndrome, and Roberts syndrome [15].

Trisomy 18 is caused by an extra copy or piece of chromosome 18. It affects 1/3,000 - 1/8,000 live births and 1/2,500 pregnancies, but the incidence goes up with maternal age [19, 20]. Less than 10% of those born with this condition will live to 1 year of age and a majority will die in less than a week after birth. Besides cystic hygromas, trisomy 18 can also include: heart defects, kidney problems, gastrointestinal issues, polyhydramnios, low-set malformed ears, clenched hands during and after pregnancy, rocker bottom feet or clubfeet, facial characteristics, delayed growth, micrognathia, microcephaly, hernias, lung anomalies, diaphragm anomalies, abdominal wall anomalies, ureter anomalies, and severe intellectual disability [14, 15, 19, 20].

Trisomy 21 (Down syndrome) is caused by an extra copy or piece of chromosome 21. Overall, it affects 1/800 live births, but the incidence goes up with maternal age [21]. Besides cystic hygromas, trisomy 21 can also affect: the heart, the central nervous system including some cognitive impairment and autistic behaviors, the gastrointestinal system, the endocrine system (hypothyroidism and diabetes), the ears and eyes, growth, and the musculoskeletal system, as well as cause leukemia [15, 21].

Trisomy 13 is caused by an extra copy or piece of chromosome 13. It affects 1/10,000 live births, but the incidence goes up with maternal age. Less than 10% of children born with this condition will live to the first year of life and 80% die before 1 month [22]. Besides cystic hygromas, trisomy 13 can also affect other systems [15, 22]. Some characteristics of trisomy 13 include: scalp defects, holoprosencephaly, cleft lip and palate, single palmar crease, polydactyly, eye and ear problems, abnormal genitalia, and severe intellectual disability. About 80% of those born with trisomy 13 have cardiovascular issues. [22].

Ten to fifteen percent of Noonan syndrome is due to an unknown cause, but the rest of Noonan syndrome is caused by mutations in one of several genes including: *PTPN11*, *SOS1*,

RAF1, and *KRAS* [21, 23]. Noonan syndrome follows an autosomal dominant pattern of inheritance, but *de novo* mutations can occur [21]. Noonan syndrome affects 1/2,500 - 1/1,000 people and characteristics of this syndrome include cystic hygromas and webbed necks [15, 24, 25]. This syndrome can also involve: a different facial appearance, congenital heart defect, short stature, minor eye problems (for 95% of individuals with this syndrome), bleeding problems, undescended testicles for males, and developmental delay. The chest shape can be affected where the nipples are widely-spaced with pectus carinatum for the upper portion of the chest and pectus excavatum for the lower portion of the chest [24, 25].

Roberts syndrome follows an autosomal recessive pattern of inheritance and is caused by mutations in the *ESCO2* gene which is located in 8q21.1 [26]. Roberts syndrome can cause cystic hygromas and hypomelia which may affect all four limbs. Hypomelia occurs when the hands and/or feet are close to the trunk of the body due to underdeveloped limb formation. Growth deficiency beginning in utero also occurs in individuals with Roberts syndrome. Craniofacial anomalies can occur including: micrognathia, microcephaly, cleft lip and palate, and an opening in the frontal section of the skull. People affected with this condition may have mild or severe intellectual disability [15, 26].

The FoxF and FoxC gene families are involved in early embryonic development specifically with the mesoderm cells. *FOXF1* and *FOXC2* genes are forkhead transcription factors in these gene families. Garabedian *et al* found that deletion of these two genes within the 16q24.1 region was associated with cystic hygroma, fetal hydrops, and a single umbilical artery on a terminated fetus (22 weeks gestation) via whole genome array comparative genomic hybridization (aCGH). Previous karyotype and FISH for chromosomes 13, 18, 21, X, and Y were normal. FISH confirmed the 16q24.1 deletion (1.1 megabases) [27]. About 50% - 60% of

fetuses with cystic hygromas will have a chromosomal condition detected by karyotype [14-17, 26]. The authors argue that whole genome aCGH will detect an additional 5.2% of chromosomal abnormalities (when copy number variants are excluded) that would not be detected by karyotype [27, 28].

1.2.6 Significance

This study aims to establish if a connection truly exists between primary lymphedema and cystic hygromas. Both primary lymphedema and cystic hygromas are caused by dysregulation of the lymphatic system so a connection between them is theoretically possible. This study will also investigate the prognosis of those affected with both lymphedema and cystic hygromas. Previous surveys sent out to participants of the Lymphedema Family Study showed 7 individuals affected with both primary lymphedema and cystic hygromas in the free response section. This project explicitly asks about cystic hygromas and lymphedema through a questionnaire. If an association exists between primary lymphedema and cystic hygromas, then this knowledge can help predict prognosis and provide appropriate care.

2.0 MATERIALS AND METHODS

2.1 DATA COLLECTION

2.1.1 Patient Population

The patient population consisted of male and female participants who have previously given consent to the Lymphedema Family Study. Inclusion criteria include those who have a diagnosis of lymphedema who have submitted biological samples in the past, and those who are related to someone with a diagnosis of lymphedema and have submitted biological samples in the past. No individual who meets the above criteria was excluded if he/she desired to participate.

2.1.2 Patient Recruitment

Only those already recruited in the past for the Lymphedema Family Study and who voluntarily submitted biological samples were contacted via mail. Depending on the responses to the questionnaire some participants were contacted with a phone call for follow-up.

2.1.3 Informed Consent

Participants have provided informed consent previously for the Lymphedema Family Study.

2.1.4 Sample

The total number of eligible participants was 1,628 which were all recruited through the Department of Human Genetics at the University of Pittsburgh.

2.1.5 Tools

An introduction letter (Appendix A) and a cystic hygroma questionnaire (Appendix B) were mailed to all of the participants. This questionnaire inquired about lymphedema status, increased nuchal thickness, nuchal edema, cystic hygroma, and septated cystic hygroma. Prognosis of these medical issues was asked in the questionnaire as well as affected family members. The Progeny database and physical records were used as tools for conducting this project, including contacting participants.

2.1.6 Questionnaire Distribution

Questionnaire distribution began in January 2015. Questionnaires were sent to participants by mail. Participants were asked to return the questionnaires within 10 days of receiving the materials.

2.1.7 Statistical Analysis

Descriptive statistics were used. Confidence intervals and Fisher's exact test were used. For the free-response sections of the questionnaire, qualitative analysis was used.

3.0 RESULTS

3.1 DESCRIPTIVES

3.1.1 Entire Sample

There are 1,628 total individuals from 247 families that make up this entire sample. These are the individuals who voluntarily donated biological samples in the past to the Lymphedema Family Study. There are 605 individuals from this sample who are affected by lymphedema (37.2%), 834 individuals without lymphedema (51.2%), and 189 individuals whose lymphedema status has not been determined (11.6%).

Out of 1,628 individuals who were mailed questionnaires, 333 were returned due to an invalid current mailing address (20.5%). Of these 333 mailings that were returned as undeliverable, 88 had email addresses listed in Progeny, and were therefore contacted via email. There were an additional 182 individuals out of the original 1,628 who did not have a complete address on file (11.2%), 60 of whom were also alternatively contacted via email.

Out of the 1,628 individuals who were mailed a questionnaire, 316 responded (19.4%) with only one who reported an increased nuchal thickness (0.3%). No respondent reported nuchal edema or a septated cystic hygroma.

3.1.2 Response Sample

There were 316 individuals out of 1,628 respondents to the questionnaire (19.4%). Out of the 316 individuals who responded: 123 replied that they have or had lymphedema (38.9%), 17 replied that they do not know if they have lymphedema (5.4%), and 176 replied that they do not have lymphedema (55.7%). Table 1 compares the data from the questionnaire respondents to the entire original sample. Data from the questionnaires of the response sample was compared to data in Progeny for the entire original sample and updates were made in Progeny for the entire original sample.

Table 1: Comparison of Lymphedema Status Between Those That Responded to the Entire Sample

| | Response Sample | Entire Sample |
|--------------------------------------|------------------------|----------------------|
| Lymphedema | 123/316 (38.9%) | 605/1,628 (37.2%) |
| No Lymphedema | 176/316 (55.7%) | 834/1,628 (51.2%) |
| Lymphedema Status Unknown | 17/316 (5.4%) | 189/1,628 (11.6%) |

- 95% Confidence Interval for Lymphedema is (0.335, 0.443) which includes the known entire sample proportion (π) of 0.372 so there is no statistically significant difference between the response sample and the entire sample. This result is desired.

- 95% Confidence Interval for No Lymphedema is (0.502, 0.612) which includes the known entire sample proportion (p_i) of 0.512 so again there is no statistically significant difference between the response sample and the entire sample. This result is desired.
- 95% Confidence Interval for Unknown Lymphedema Status is (0.029, 0.079) which does not include the known entire sample proportion (p_i) of 0.116 so there is a statistically significant difference between the response sample and the entire sample. This result is not desired.

Overall, the response sample is representative of the entire sample with the exception of the Unknown Lymphedema Status proportions. These confidence intervals were used to check for potential bias of those who responded.

3.2 QUALITATIVE

3.2.1 Cystic Hygroma and Nuchal Thickness

As stated in the Introduction, there were 7 individuals mentioned in the free response section of the original Lymphedema Family Study survey who had lymphedema and cystic hygromas plus another individual with lymphedema that may have had a cystic hygroma. This was the inspiration for this project. Table 2 shows this data as well as increased nuchal thickness (NT), nuchal edema (NE), and nuchal fold (NF) for fifteen individuals.

Table 2: Original Lymphedema, Cystic Hygroma, and Nuchal Data

| Individual | Phenotype | Lymphedema Affection Status |
|----------------------|--------------------------------------------|-----------------------------------------------------------------------------------------|
| 1 | CH and Fetal Hydrops: Not Turner Syndrome* | Affected |
| 2 | Subdural Hygroma (not CH) | Affected |
| 3 | CH and NT | Affected |
| 4 | NT | Affected |
| 5 | NF | Unaffected (current age 19) Average age of onset for family (7.9) |
| 6 | CH | Affected |
| 7 | CH? Noonan's? | Indeterminate (age at original survey - 6) Average age of onset for family (16.7) |
| 8 | Distichiasis and NF | Affected |
| 9 | CH | Affected |
| 10 | CH | Affected |
| 11 | NT/CH | Affected |
| 12 | CH/hydrops* | Affected |
| 13 (sibling to # 14) | NT | Indeterminate (miscarriage) Average age of onset for family (13.2) |
| 14 (sibling to # 13) | NT | Indeterminate (current age 2) Average age of onset for family (13.2) |
| 15 | NT to CH to hydrops* | Affected |

*Individuals captured on original survey and cystic hygroma questionnaire

Following are the replies to the cystic hygroma questionnaire.

Family A replied back “Child Female Did Not Survive Birth With Turner’s Syndrome”. This family was not able to be contacted to verify lymphedema or cystic hygroma. She was diagnosed at 19 weeks of gestation and was stillborn. Her brother has lymphedema since birth. No other family members have lymphedema. The pedigree for this family is in Appendix C.

An individual from Family R states, “My brother,..., had a child with a thick neck detected in utero. At birth it was resolved.” Lymphedema or cystic hygroma status was unknown due to failure to contact family. The individual that had a “thick neck” is now 12-14 years old with no lymphedema. There is a hereditary pattern of lymphedema with three generations affected including the father of the child with a “thick neck”. The average age of onset of lymphedema for this family is 22.4 years. The pedigree for this family is in Appendix D.

Family G stated “Nuchal thickness in utero one baby that did not survive birth (miscarriage at 17-18 weeks). In utero, my daughter (current age 10) had increased nuchal thickness noted as part of the Down Syndrome test but has never been tested or noted as a problem since. She is ‘very normal’ ”. No genetic testing was done for these children and neither one has been diagnosed with lymphedema. Four generations affected with lymphedema, but not the mother (age 50) of these two children. The average age of onset of lymphedema for this family is 28 years. The pedigree for this family is in Appendix E.

One 45 year old man from Family P commented on his own lymphedema (which did not increase or decrease over time), genetic test results in the family, and cystic hygroma in the family. This is what he wrote with respect to himself. “Lymphedema onset at puberty, approximately age 15. (Distichiasis present since childhood)...Lymphedema-distichiasis syndrome has not severely impacted my quality of life, but it has frequently been cause for

discomfort or for worry over peer acceptance, health, and safety. It regularly affects my choice of clothing. It has twice caused great illness when infections entered my body through my feet.” This is what he wrote down for his family. “My maternal grandmother..., mother..., and sister... have lymphedema-distichiasis syndrome, and have had the *FOXC2* gene mutation identified through this U of Pittsburgh lymphedema family study. Most importantly, my 3-year-old son, [R for confidentiality], displayed cystic hygroma in utero. My wife’s...OB/GYN office discovered it on the back of [R’s] neck during a routine ultrasound when she was pregnant, prompting them to refer our case to a high-risk perinatal office ... and starting what were some very scary months for us in late 2010 and early 2011. Three months after it was discovered on [R’s] neck, the cystic hygroma was discovered to be receding. When [R] was born on June 29, 2011, no remnant of the cystic hygroma was visible, though there was a little bit of excess skin on the back of his neck. That excess skin disappeared as [R] grew.” A sister of the man who wrote this also replied back to the questionnaire. She describes her own lymphedema which has not increased or decreased over time. “Age 30, not related to pregnancy, Location: calves, ankles and feet. I believe it was triggered by a very long airplane flight.” And she wrote about her daughter. “My daughter (15) has not been officially diagnosed, nor does she presently show swelling, but she has the trademark family ingrown eyelashes. When she was born she had extra skin at the back of her neck, but there was no cause for concern and it went away as an infant.” There is a *FOXC2* mutation in this family and it is an insertion of cytosine at base-pair position 609 (c.609insC) which yields a protein with a termination at amino acid position 463 (p.463X). Individuals in this family with this mutation are the 45 year old man, his mother, and his maternal grandmother. This exact mutation in this family was found by Finegold and Ferrell et al. in 2001 under “Family F” in the journal article. “Family F” in this journal article is “Family

P” for this questionnaire study [29]. It is important to note that four generations of this family are affected by lymphedema-distichiasis. The average age of onset of lymphedema for this family is 17.6 years. The pedigree for this family is in Appendix F.

The mother in Family C noted that one of her sons (C) has Milroy’s disease (primary lymphedema) since birth and that her other son (P) had non immune hydrops and cystic hygroma. C also has an 8p11.23 deletion. P was stillborn at 27 weeks after an intrauterine fetal demise. He was also diagnosed with lymphedema, but had a normal karyotype and microarray. No other family members have lymphedema. This individual was already captured on the older survey and is individual 12 in Table 2. The pedigree for this family is in Appendix G.

Family J exhibits five generations affected with lymphedema. One individual mentioned her own lymphedema (did not increase or decrease with time) diagnosis as well as other family members. “Primary lymphedema dx approx. 1998 at age 27. Both lower limbs with some abdominal involvement. Also I have distichiasis as do my 2 daughters. I started showing symptoms at age 14 and they increased after my first pregnancy (the stillborn with cystic hygroma). It worsened until my diagnosis and treatment at 27. It has affected my quality of life by reducing the activity level I’m able to enjoy with my family. Also, it is somewhat emotionally draining and taxing on a person’s self-esteem. I had a stillborn baby girl in 1992 at 28 weeks gestation. She was diagnosed with cystic hygroma with fetal hydrops as the cause of death.” The girl who was stillborn also had a diagnosis of lymphedema. An amniocentesis was done during pregnancy and she did not have 45,X (Turner syndrome). This individual was already captured on the previous survey. She is Individual 1 in Table 2. The mother did have genetic testing for herself and she has a *FOXC2* mutation (c.377T>C, p.L126P). Although both of her daughters have distichiasis, only the elder (age 21) has developed lymphedema by the time

of this survey. The younger daughter is 17 years old. The average age of onset of lymphedema for this family is 17.1 years. The pedigree for this family is in Appendix H.

The mother in Family K wrote that “My daughter ([L] - enrolled) was diagnosed with increased NT at 13 weeks that quickly progressed to a cystic hygroma and fetal hydrops. She lived till 23 weeks” of gestation. L was also noted to have pleural effusion, ascites, lymphangiectasia, and hydronephrosis. She was conceived through her parents’ gametes via in-vitro fertilization. L, her father, her paternal uncle, and her paternal grandfather were all diagnosed with lymphedema with a *FOXC2* mutation present in both L and her father (c.223insT; p.Tyr75Leu fs*388). L has been captured on the previous survey. She is individual 15 in Table 2. The average age of onset of lymphedema for this family is 14.4 years. The pedigree for this family is in Appendix I.

3.3 TESTING FOR SIGNIFICANCE

3.3.1 Testing for Significant Difference of Cystic Hygroma Prevalence Between Those With and Without Lymphedema (Aims 1 and 2)

There are three people with cystic hygromas out of 123 with lymphedema (2.44%). There is also one person with cystic hygromas out of 176 without lymphedema (0.57%). Fisher’s exact test was used to examine if there was a statistically significant difference of cystic hygroma prevalence between those with and without lymphedema. This test yielded a p-value of 0.3094. Since the alpha level is set at 0.05, this means that the difference seen was not statistically significant.

4.0 DISCUSSION

4.1 AIM 1: ASCERTAINING THE PROPORTION OF PEOPLE WITH CYSTIC HYGROMAS OF THOSE WITH AND WITHOUT LYMPHEDEMA

This Aim was achieved with data from the cystic hygroma questionnaire. Of the 123 surveys sent back from individuals with lymphedema, 3 also had a history of cystic hygromas (2.44%). Of the 176 surveys returned from individuals without lymphedema, 1 also had a previous cystic hygroma (0.57%). If one was to include increased nuchal thickness due to the one case that had an increased nuchal thickness develop into a cystic hygroma (Family K), then there were 4 cases of cystic hygroma/increased nuchal thickness (1 cystic hygroma + 3 increased nuchal thickness) out of 176 people without lymphedema (2.27%). “Thick neck” or thickened skin at the back of the neck were not included because these descriptions are not diagnostic, but can be suggestive of past cystic hygroma.

4.2 AIM 2: TO DETERMINE IF A STATISTICAL SIGNIFICANT DIFFERENCE EXISTS IN CYSTIC HYGROMA PREVALENCE BETWEEN THOSE WITH AND WITHOUT LYMPHEDEMA

The Fisher's exact test was performed to achieve Aim 2. Based on a p-value of 0.3094 which is greater than the alpha level of 0.05, it is determined that the difference in cystic hygroma prevalence between those with (2.44%) and without lymphedema (0.57%) is not statistically significant. With only three confirmed cases of cystic hygroma with lymphedema and one confirmed case of cystic hygroma without lymphedema, this result is not unexpected due to the low numbers of individuals affected with cystic hygroma. In addition, it was not useful to include increased nuchal thickness cases, since these were only reported among the individuals without lymphedema (2.27%) which only serves to weaken the significance of this result. In this situation, Fisher's exact test would yield a p-value of 1.0 meaning that there was absolutely no statistical significant difference in cystic hygroma/increased nuchal thickness prevalence between those with and without lymphedema.

It should be noted that the Fisher exact test used may be flawed in this situation. The one individual who was diagnosed in utero with cystic hygroma, but was not diagnosed with lymphedema may develop lymphedema in the future because he is only three years old and the average age of onset for lymphedema in this family (Family P) is 17.6 years. He has a 50% chance of inheriting the *FOXC2* mutation from his father and may develop lymphedema in the future.

The three individuals who have been diagnosed with both lymphedema and cystic hygromas (all died before birth) make up 2.44% of those respondents with lymphedema, but the general population prevalence of cystic hygromas for miscarriages is 1/750 or 0.1333% (18.3-

fold difference) [14]. This greatly strengthens the argument that there should be a higher prevalence of cystic hygromas for those with lymphedema compared to those without lymphedema since 18.3-fold difference is a big difference.

4.3 AIM 3: TO ASCERTAIN THE PROGNOSIS OF PEOPLE WITH CYSTIC HYGROMAS AND LYMPHEDEMA

The only individuals from the cystic hygromas questionnaire with both cystic hygromas and lymphedema were the three babies that died in utero. One of the three cases that had both cystic hygroma and lymphedema in utero had increased nuchal thickness at 13 weeks which developed into a cystic hygroma and fetal hydrops (Family K). She and her father both harbored a *FOXC2* mutation. The average age of onset of lymphedema for family K is 14.4 years. One of the other lymphedema/cystic hygroma cases has a mother with a missense *FOXC2* mutation (Family J). The average age of onset of lymphedema for this family is 17.1 years. In family C, the son who died in utero (had both cystic hygroma and lymphedema) has a brother who is currently 4 years old who has Milroy's disease since birth. No other family members in family C have lymphedema. All three cases that had both cystic hygromas and lymphedema were previously captured on the earlier survey in the free response section that was the inspiration for this project.

There was one case of cystic hygroma that did not develop lymphedema in utero, but he is currently 3 years old and is from a family with significant primary lymphedema and a known truncating *FOXC2* mutation (Family P). It is possible that he did not inherit the *FOXC2* mutation (no genetic testing was performed on him), but the possibility of developing lymphedema (specifically lymphedema-distichiasis) in the future cannot be ruled out. His cystic

hygroma disappeared as well as the thick skin at the back of his neck. His cousin was born with extra skin at the back of her neck and has distichiasis, but does not have lymphedema currently. She is now 15 years old. The average age of onset of lymphedema for this family is 17.6 years. Lymphedema-distichiasis typically has onset during puberty (not congenital) [4, 8].

Family G did have one child who died in utero after being diagnosed with an increased nuchal thickness, but the mother also has a daughter who is currently 10 years old who had an increased nuchal thickness in utero. The average age of onset of lymphedema for this family is 28 years so the daughter who is currently 10 years old may develop lymphedema in the future.

For people with lymphedema, the common responses to the questionnaire were pain, swelling, difficulty finding the right clothes, and difficulty with mobility. Some have reported being embarrassed or having their own self-esteem or self-body image harmed. There were some who reported not being affected that much by the lymphedema.

4.4 IMPLICATIONS AND PUBLIC HEALTH SIGNIFICANCE

Lymphedema is an incredibly complex disease that can have significant burdens both physically and psychologically. Cystic hygromas can cause significant cosmetic and medical complications including miscarriages and stillbirths. Approximately 50-60% of individuals with cystic hygromas have a chromosomal or genetic syndrome that also makes medical management and treatment more complex [14, 15, 17, 18]. Both lymphedema and cystic hygromas are medical issues involving the lymphatic system. The main objective of this project was to determine if an association exists between cystic hygromas and lymphedema, the presence of which may help

predict prognosis and provide appropriate care. There is some evidence that this association exists, but it is not statistically significant.

4.5 LIMITATIONS

One limitation of this project was the low response rate to the cystic hygroma questionnaire (19.4%). Participating in this questionnaire was voluntary. A strength of this project; however, was that those who did respond were representative of the lymphedema affection status of the original sample.

Another limitation of this project was the lack of access to medical records, both paper and electronic, except for what has been previously provided to us by the participants themselves. One difficulty of a retrospective study is that participants may not have access to previous medical records or may not recall certain medical information. Medical records may contain information that participants and their family members do not know or remember. Participants of the Lymphedema Family Study come from all over the United States and all over the world. The United States is moving toward an all-electronic medical record system which may not be accessible through Progeny in the future due to privacy concerns including H.I.P.A.A. Many paper medical records may not exist anymore due to destruction.

Since the Lymphedema Family Study is 20 years old, there is a high probability that many participants have moved, resulting in invalid contact information stored in Progeny. In fact, 20.5% of cystic hygroma questionnaires were returned as undeliverable. Although attempts were made to contact these individuals via email, as well as those whose addresses were not

listed in Progeny (11.2%) - only 28.7% of these individuals had an email address listed in Progeny.

Another limitation of this project was that the only cases that had both cystic hygromas and lymphedema were cases that were already captured in the original survey for the Lymphedema Family Study, yet only three of these were reacquired for this project. The low numbers of increased nuchal thickness and cystic hygromas for both those with and without lymphedema resulted in data not reaching statistical significance.

One reason for the low numbers of cystic hygroma cases could be due to underreporting. Perhaps more individuals had a cystic hygroma in utero, but were not diagnosed because the screening technology was not readily available at the time. Prenatal ultrasounds may not have existed for many of the participants or they may not have been performed for most individuals included in the original study. Offering prenatal ultrasounds as standard-of-care, particularly first trimester screening is a relatively recent phenomenon, but one that will likely continue to grow with time.

4.6 FUTURE RESEARCH

The best way to truly determine if an association exists between primary lymphedema and cystic hygromas is to have a multi-year, multi-site prospective study. If cost is a major concern, then this study can be done on families with lymphedema only. This study would be performed at medical institutions that offer prenatal ultrasounds as standard-of-care. Those that have been diagnosed with having a cystic hygroma in utero would be followed regardless of pregnancy outcome (miscarriage, stillbirth, or live birth). For those that are live birth, they would be

followed for many years in order to maximize the chance that lymphedema diagnoses would be recorded. This would be an ambitious project, but could potentially aid future individuals with cystic hygromas due to any cause, including hereditary lymphedema.

APPENDIX A: INTRODUCTION LETTER



University of Pittsburgh

Department of Human Genetics

Graduate School of Public Health
A300 Crabtree Hall
130 DeSoto Street
Pittsburgh, PA 15261
412-624-3018
Fax: 412-624-3020

December 4, 2014

Dear Participant,

Thank you for your previous participation in the University of Pittsburgh's Lymphedema Family Study. You are receiving this letter for a very important reason, to request your continued participation in the Lymphedema Family Study by completing a short addendum to the Lymphedema Questionnaire that you previously completed, this time regarding your personal or family history of cystic hygroma. Cystic hygroma is a collection of lymphatic fluid at the back of the neck that may be observed during a prenatal ultrasound exam and then disappear on its own, though it can sometimes still be present at the time of birth. There are many potential causes of cystic hygroma, but there is some evidence that one cause of cystic hygroma is inherited lymphedema. Enclosed is a short questionnaire to investigate the connection between lymphedema and cystic hygroma. Filling out this questionnaire is completely voluntary and the confidentiality of your responses will be strictly maintained. If you choose to complete this questionnaire, which also requests updated contact information, please return it by mail (Attn: Kala Kennedy) or fax to my attention at your earliest convenience, but no later than January 16, 2015. You may also receive a follow-up phone call for clarification or additional information regarding any positive responses you provide.

Please feel free to contact me with any questions or concerns at (412) 624-4659 or by email at genetics@pitt.edu. The questionnaire can also be faxed to my attention at (412) 624-3020. Please refer to our web page for additional information about the research study or lymphedema in general. Go to www.lymphedema.pitt.edu and click on "Research Update" for the most current information about the Lymphedema Family Study. Advances in understanding the underlying causes of lymphedema are critically dependent on the participation of individuals and families such as yours in our research, so we would like to take this opportunity to thank you again for your interest and participation in the Lymphedema Family Study.

Sincerely,



Joshua Carpenter, B.S.
Genetic Counseling Intern



Dr. David Finegold, M.D., Ph.D.
Professor of Pediatrics and Human Genetics

enclosures

APPENDIX B: CYSTIC HYGROMA QUESTIONNAIRE

Date Completed: _____

For Study Use Only: Study ID#: _____

QUESTIONNAIRE ADDENDUM: LYMPHEDEMA AND CYSTIC HYGROMA Please provide updated contact information below.

Name: _____ Gender: ☐ Male ☐ Female Birth Date: ____/____/____

Address: _____

Phone (Home) _____ (Work) _____ (Cell) _____

Best Time To Call? _____ E-Mail Address: _____

Contact Preferences? Check all that apply: ☐ Home No. ☐ Work No. ☐ Cell No. ☐ E-mail ☐ Mail

Have you ever been diagnosed with having: Yes No Don't Know

| | | | |
|----------------------------|--------------------------|--------------------------|--------------------------|
| Lymphedema | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Increased Nuchal Thickness | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Nuchal Edema | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Cystic Hygroma | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Septated Cystic Hygroma | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

If you answered "yes" to any of the above questions, please provide additional details below for each "yes" such as age at diagnosis (including week of pregnancy for fetal diagnoses) and body location of swelling:

Please complete the following statements by only checking ONE box per statement regarding the current status of each diagnosis.

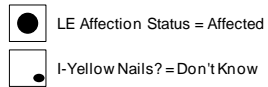
| | Disappeared | Decreased | Remained the Same | Increased | N/A |
|------------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| My lymphedema has: | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| My increased nuchal thickness has: | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| My nuchal edema has: | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| My cystic hygroma has: | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| My septated cystic hygroma has: | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

If you answered anything other than "N/A" above, please provide additional details for each about how old you were when this occurred (including week of pregnancy for fetal diagnoses) and how this has affected your quality of life:

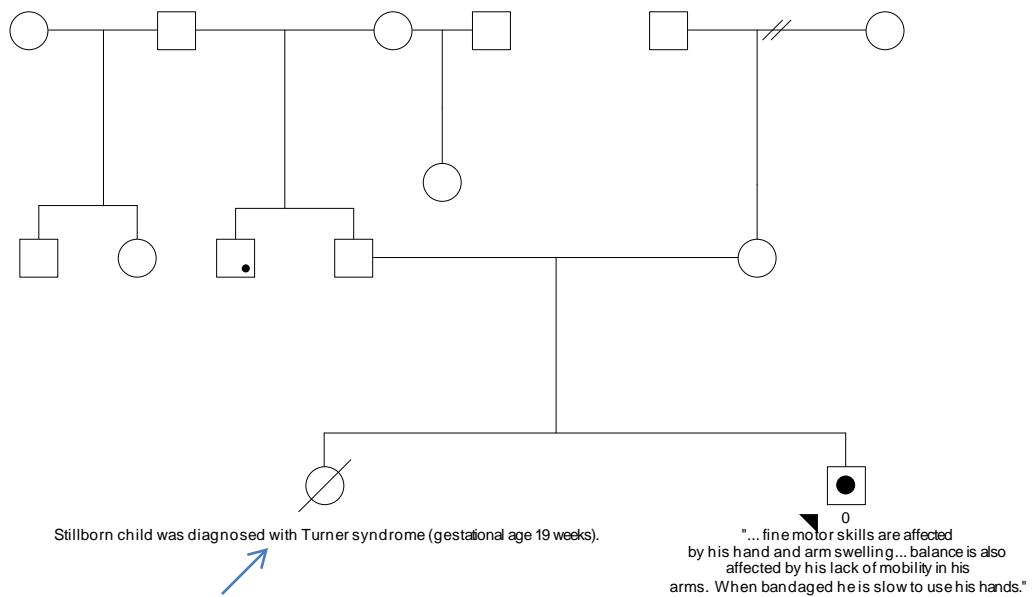
Has anyone else in your biological family been diagnosed with any of these conditions, including any babies that did not survive to birth? If yes, please indicate their relationship to you and provide details about their condition(s).

* Please continue on the back of this page if more space is necessary for any of the questions on this form.

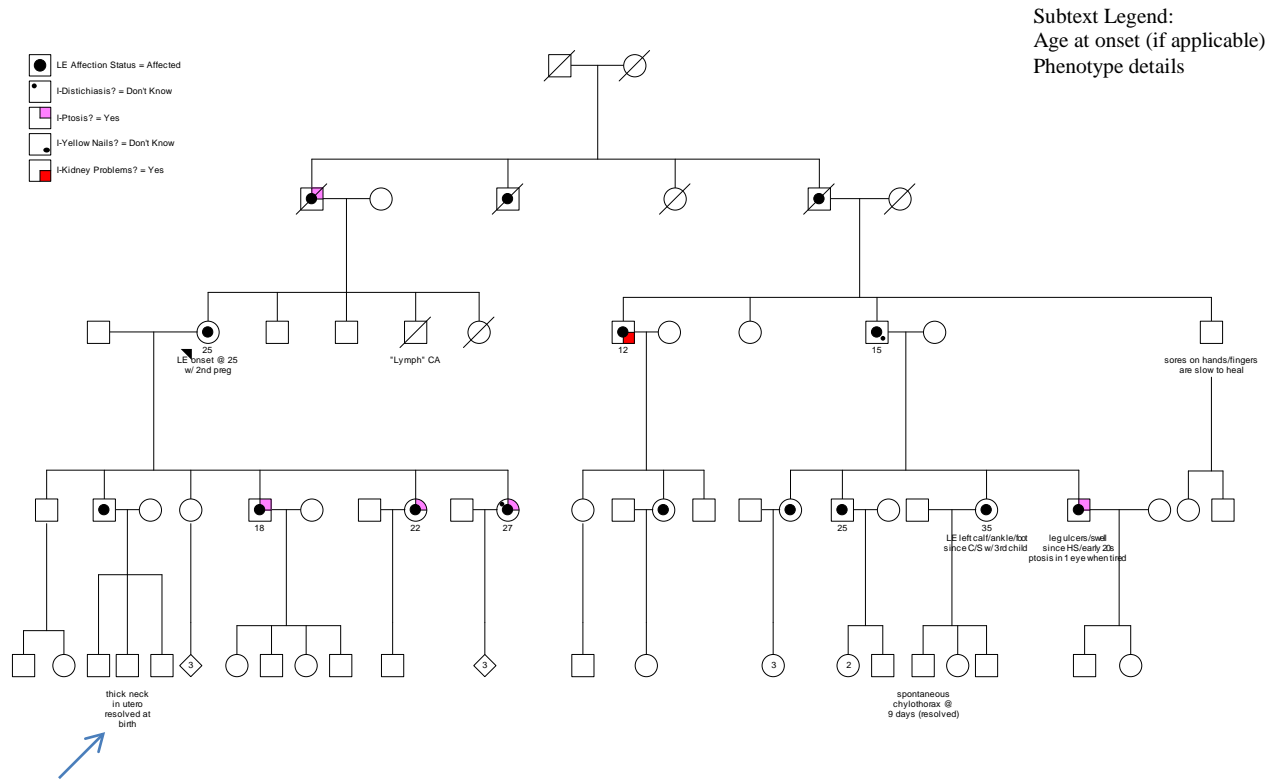
APPENDIX C: FAMILY A PEDIGREE



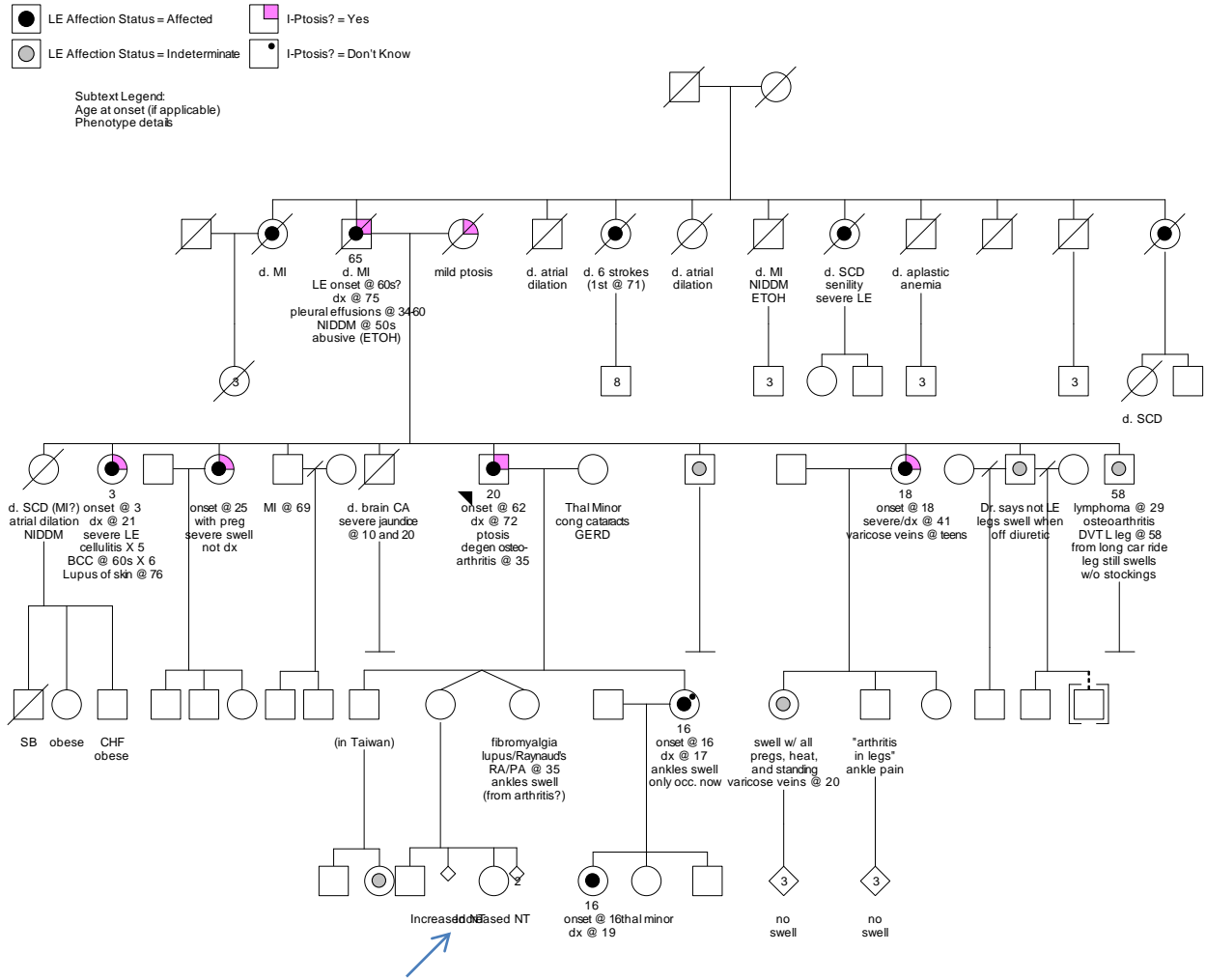
Subtext Legend:
 Age at onset (if applicable)
 Phenotype details



APPENDIX D: FAMILY R PEDIGREE



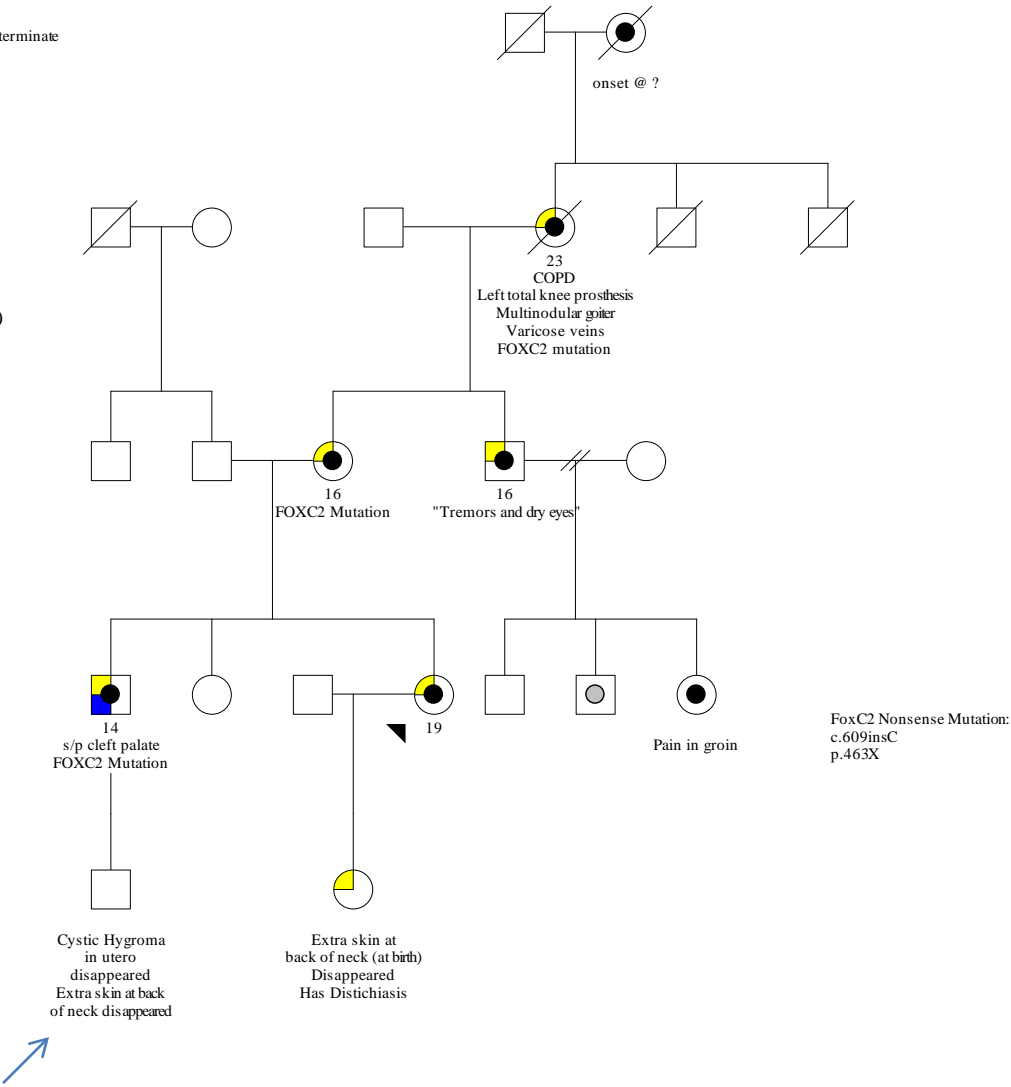
APPENDIX E: FAMILY G PEDIGREE



APPENDIX F: FAMILY P PEDIGREE

- LE Affection Status = Affected
- LE Affection Status = Indeterminate
- I-Distichiasis? = Yes
- I-Cleft Palate? = Yes

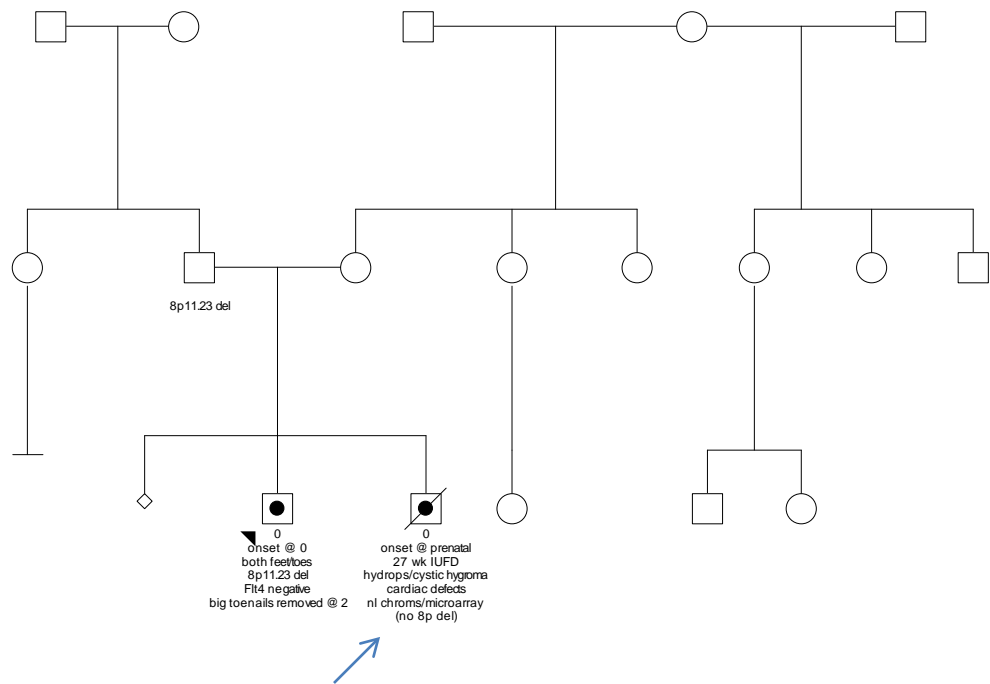
Subtext Legend:
Age at onset (if applicable)
Phenotype details



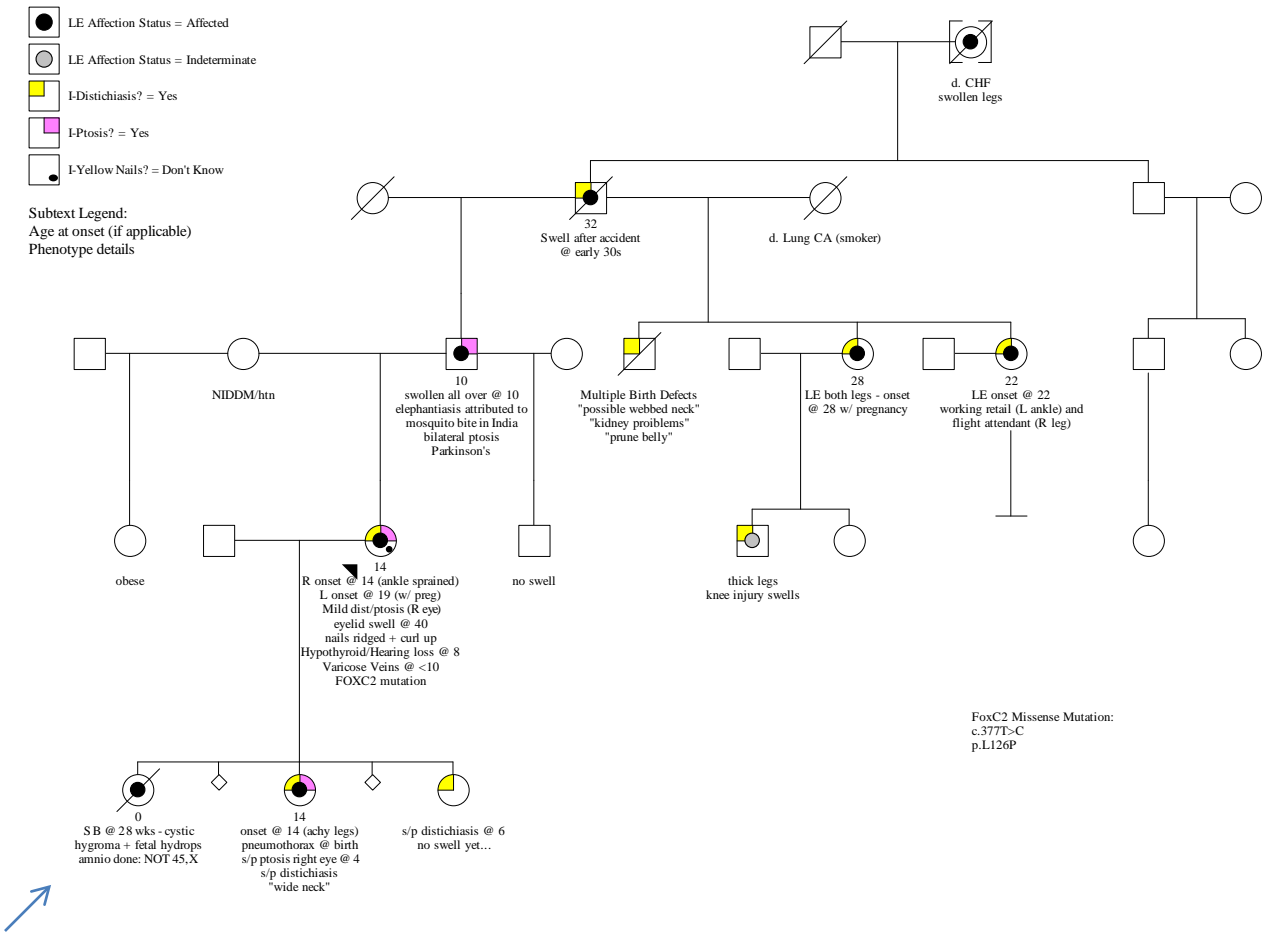
APPENDIX G: FAMILY C PEDIGREE

 LE Affection Status = Affected

Subtext Legend:
 Age at onset (if applicable)
 Phenotype details



APPENDIX H: FAMILY J PEDIGREE

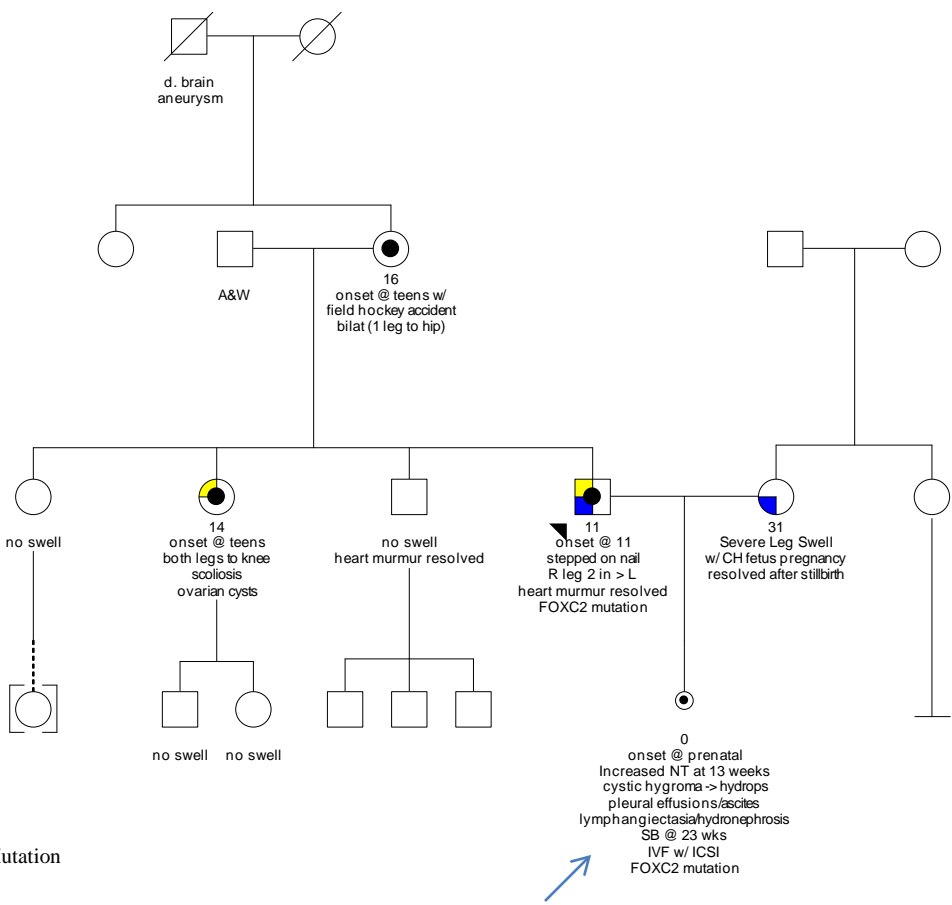


APPENDIX I: FAMILY K PEDIGREE

- LE Affection Status = Affected
- I-Distichiasis? = Yes
- I-Heart Defect? = Yes

Subtext Legend:
Age at onset (if applicable)
Phenotype details

FoxC2 Frameshift Mutation
c.223insT
p.Tyr75Leu fs*388




APPENDIX J: IRB APPROVAL LETTER

PI Notification: Your requested expedited modific... - Carpenter, Josh... <https://outlook.office365.com/owa/projection.aspx>

PI Notification: Your requested expedited modification has been approved

IRB
Fri 12/5/2014 4:29 PM
Inbox
To: Carpenter, Joshua Mark <JMC256@pitt.edu>;



University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Ground Level
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
<http://www.irb.pitt.edu>

Memorandum

To: David Finegold, MD
From: IRB Office
Date: 12/5/2014

IRB#: MOD960639-12 / IRB960639
Subject: Genetic Studies of Lymphedema

The University of Pittsburgh Institutional Review Board reviewed and approved the requested modifications by expedited review procedure authorized under 45 CFR 46.110 and 21 CFR 56.110.

Modification Approval Date: 12/5/2014
Expiration Date: 7/9/2015

For studies being conducted in UPMC facilities, no clinical activities that are impacted by the modifications can be undertaken by investigators until they have received approval from the UPMC Fiscal Review Office.

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. Refer to the IRB Policy and Procedure Manual regarding the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

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